A novel model for prognosis of Meniere's disease using oxidative stress susceptibility of lymphoblastoid cell lines

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Summary

The aim of this study was to examine differences of susceptibility to oxidative stress of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) established from Meniere's disease (MD) patients and to examine the effect of ATP treatment in the prognosis and treatment of MD. LCLs were established from 10 patients with MD and 10 healthy donors by EBV. Cell viabilities were calculated after treatment of H₂O₂ with or without ATP. The relationship between the sensitivity of H₂O₂-treated LCLs to ATP and the staging scale of MD was examined. The nuclear morphological changes of Hoechst 33258-stained LCLs after H₂O₂-treatment were observed under a fluorescence microscope. LCLs from MD were significantly more sensitive (p < 0.001) to H₂O₂ than LCLs from healthy donors after 3 h of H₂O₂ treatment. All of the ATP-sensitive LCLs were categorized as Stage 1 or 2, while others categorized as Stage 3 or 4 were not sensitive to ATP. There were significant differences (p < 0.01) of cell viabilities after addition of ATP between H₂O₂-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 in MD. Both chromatin condensation and swelling of the cell body were observed in H₂O₂-treated LCLs. Our findings indicate that LCLs established from MD patients might be used as a unique model to detect susceptibility to oxidative stress and ATP treatment in MD patients. Also, the difference of the sensitivity of H₂O₂-treated LCLs to ATP might relate to prognosis and treatment of MD. This system may form the basis of tailor-made therapy for MD.

Keywords: Meniere's disease (MD), lymphoblastoid cell line (LCL), oxidative stress, adenosine 5'-triphosphate (ATP)

1. Introduction

Meniere's disease (MD), described by Prospero Meniere in 1861, is typically characterized by fluctuating hearing loss, episodic vertigo, tinnitus and a sensation of pressure. The histopathological hallmarks of the disease, at the bone level, are endolymphatic hydrops, atrophy and erosion of the endolymphatic sac. Despite a rigorous pathological definition, the etiology of MD, which is usually defined as idiopathic, is ascribed to a variety of causes, such as alterations of ionic homeostasis, vascular disorder, trauma, viral infections and immunological disorder. However, until recently it has been difficult to estimate the prognosis of MD clinically. Current studies have reported that oxidative stress may play a crucial role in the pathogenesis of a variety of inner ear diseases, such as noise-induced hearing loss (1), ischemic impairment (2) and age-related hearing loss (3). Concerning MD, Horner and Guilhaume suggested that oxidative insult was likely to contribute to the pathology associated with endolymphatic hydrops and therefore free radical scavengers might be useful in the treatment of MD patients (4). Takumida et al. demonstrated that edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), an
inhibitor of reactive oxygen species (ROS), attenuated the formation of endolymphatic hydrops in the guinea pig cochlea (5). In a clinical trial, treatment using such radical scavengers was reported to have the potential to become an effective new therapy for patients with MD (6). However, the direct effect of oxidative stress for MD inner ear tissue cells is still unknown, because the normal inner ear tissue can not be obtained. Therefore, we focused on Epstein-Barr virus (EBV)-transformed B-lymphocytes (lymphoblastoid cell lines; LCLs) as a cellular model for MD like hypertension (7), diabetes mellitus (8), Alzheimer's disease (9), Huntington's disease (10), and bipolar disease (11), because we hypothesized that LCLs could be used in place of inner ear cells of patients with MD. In addition, LCLs can be easily established from B-lymphocytes obtained from patients using EBV infection and can be maintained for a long time.

Hydrogen peroxide (H$_2$O$_2$) is an intermediate product of the degradation of ROS and a highly reactive molecule. The treatment of cells with H$_2$O$_2$ induces oxidative stress via an increased production of ROS and may subsequently lead to cell damage or cell death. Extracellular H$_2$O$_2$ is able to cross cell membranes and directly alters their intracellular concentration (12). The loss of adenosine 5'-triphosphate (ATP) has been reported to be an early step after initiation of H$_2$O$_2$-induced oxidative stress in non-neuronal (13) and neural systems (14). Teepker et al. reported that ATP-decline under H$_2$O$_2$-induced oxidative stress might point to a relevant ATP consumption related to apoptosis (15). In this study, we evaluated the difference of the susceptibility of the LCLs to H$_2$O$_2$-induced oxidative stress from patients with MD and healthy donors. We also investigated the ability of ATP treatment to modulate the cell viability of LCLs loaded with H$_2$O$_2$ and considered the relationship between the sensitivity of the H$_2$O$_2$-treated LCLs to ATP and the staging scale of MD. The aim of this study was to examine whether the difference of the susceptibility of LCLs to H$_2$O$_2$-induced oxidative stress and the effect of ATP treatment reflects the prognosis of MD. To our knowledge, this is the first report in which LCLs established from the patients were used as a cellular model for MD.

2. Materials and Methods

2.1. Materials

Ten patients with MD as defined by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) (4 males and 6 females, 54.7 ± 13.7 years old) and 10 healthy volunteers (5 males and 5 females, 53.5 ± 11.4 years old) were studied. Informed consent was obtained from all cases. Sensory hearing threshold was classified on the four-way classification of the American Academy of Otolaryngology (Committee on Hearing and Equilibrium, 1995): Stage 1 (mean threshold < 26 dB), Stage 2 (mean threshold 26-40 dB), Stage 3 (mean threshold 41-70 dB), and Stage 4 (mean threshold > 71 dB). Mean threshold was in each case calculated as the arithmetic mean of the threshold at 4 frequencies (500, 1,000, 2,000, and 3,000 Hz) measured on the same day as the dynamic posturography session.

2.2. Cell Culture

Peripheral blood lymphocytes (PBMC) were obtained from patients with MD and normal controls and transformed by Epstein-Barr virus (B95-8 strain) for establishing LCLs as described elsewhere (16,17). The LCLs were grown in complete medium consisting of RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10 % fetal calf serum (FCS).

2.3. Viability assay

Cells were suspended at a density of 4.0 × 10$^6$ cells/mL in complete medium and seeded at 1.0 × 10$^4$ cells per well of a 96-well UV plate (Nunc, Roskilde, Denmark). An equal volume of 0.04 mM H$_2$O$_2$ was added to each cell suspension and the mixtures were incubated at 37°C. The adequate concentration of H$_2$O$_2$ in this study was 0.02 mM at the final concentration by determining the results of the preliminary experiments and on the basis of the effects of H$_2$O$_2$ in HeLa cells (18), fibroblasts (19), cardiac myocytes (20), or human T-lymphoma (21). ATP (GE Healthcare, Salt Lake City, UT, USA) was also added as 20× stock solution to 5 mM at the final concentration. Cell viability was determined by trypan blue exclusion. Cells that were treated with H$_2$O$_2$ with or without ATP and incubated at 37°C for the times indicated were suspended in an equal volume of 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA). Dead (blue) and live (clear) cells were counted using a hemocytometer. The percentage of viability was defined as the number of live cells divided by the number of live and dead cells. All experiments were performed in triplicate.

2.4. Morphological examinations of cells

LCLs exposed to the medium containing 0.02 mM H$_2$O$_2$ for 5 h were observed under a phase-contrast microscope. For determination of nuclear morphological change, the cells were additionally incubated with 1 μL of Hoechst 33258 (Dojindo, Kumamoto, Japan) (1 mg/mL) for 10 min. After staining, the cells were washed with PBS and analyzed under a fluorescence microscope.

2.5. Statistical Analysis

All statistical analysis was performed using Ystat 2004.
Effects of ATP addition on H$_2$O$_2$ cell damage in LCLs

Figure 2 showed that the viability after 5 h of 0.02 mM H$_2$O$_2$ treatment were 24.6% in Me1 cells, 3.3% in Me2 cells, 27.8% in Me3 cells, 8.2% in Me4 cells, 5.9% in Me5 cells, 0% in Me6 cells, 5.6% in Me7 cells, 9.4% in Me8 cells, 4.3% in Me9 cells, and 22.2% in Me10 cells. Addition of ATP could obviously recover the viabilities of the H$_2$O$_2$-treated LCLs from Me1, Me3, Me4, Me7, and Me10 patients (Figures 2A, 2C, 2D, 2G, and 2J). In contrast, the viabilities of H$_2$O$_2$-treated LCLs from Me2, Me5, Me6, Me8, and Me9 patients decreased in a similar manner as when ATP was not added (Figures 2B, 2E, 2F, 2H, and 2I). The viabilities after 5 h of treatment of 0.02 mM H$_2$O$_2$ and the addition of 0.05 mM ATP were 89.9% in Me1 cells, 7.2% in Me2 cells, 84.8% in Me3 cells, 61.1% in Me4 cells, 10.0% in Me5 cells, 0% in Me6 cells, 80.1% in Me7 cells, 7.8% in Me8 cells, 4.4% in Me9 cells, and 79.3% in Me10 cells. These data indicate that Me1, Me3, Me4, Me7, and Me10 cells might be sensitive to ATP. On the other hand, Me2, Me5, Me6, Me8, and Me9 cells are not as strongly affected by ATP.

The relationship between the sensitivity of H$_2$O$_2$-treated LCLs to ATP and the staging scale of MD

As shown in Figure 3, the ATP-sensitive LCLs (Me1, Me3, Me4, Me7, and Me10) were classified as Stage 1 or 2, while the ATP-insensitive LCLs (Me2, Me5, Me6, Me8, and Me9) were classified as Stage 3 or 4. There were significant differences ($p < 0.01$) in the viabilities of H$_2$O$_2$-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 in MD after the addition of ATP.

Effects of H$_2$O$_2$ on membrane integrity and chromatin structure

Both chromatin condensation and swelling of the cell body were observed after treatment with 0.02 mM H$_2$O$_2$ (Figure 4).

Discussion

Treatment of cells with H$_2$O$_2$ induces oxidative stress, accompanied by lipid peroxidation, DNA and protein damage (22), and finally cell death (23). In addition, oxidative stress is able to disturb cellular energy metabolism as a result of the decrease of ATP in a variety of cells (24). These studies were based on the hypothesis that the susceptibility of individual cells to oxidative stress was different from one person to another. Our results strongly demonstrated that LCLs from patients with MD were significantly more sensitive ($p < 0.001$) to oxidative stress than LCLs from healthy donors.
Figure 2. Effect of ATP addition to the viabilities of LCLs after H2O2 treatment. Addition of ATP could obviously recover the viabilities of H2O2-treated LCLs from Me1, Me3, Me4, Me7, and Me10 patients (A, C, D, G, and J, respectively). In contrast, the viabilities of H2O2-treated LCLs from Me2, Me5, Me6, Me8, and Me9 patients decreased in a similar manner as when ATP was not added (B, E, F, H, and I, respectively).
from healthy donors (Figure 1). In other words, LCLs from healthy donors were resistant to H₂O₂, while LCLs from MD patients were not. This finding also suggests that patients with MD can be diagnosed by the difference of susceptibility of established LCLs to oxidative stress.

Next, we investigated the effect of ATP treatment on H₂O₂-treated LCLs. At an early stage of cell damage, ATP-depletion and intracellular Ca²⁺ alteration may occur under H₂O₂-induced oxidative stress (25). The concentration of extracellular ATP regulates various signaling systems including propagation of intercellular Ca²⁺ signals. To reveal the cause of ATP-depletion after the exposure of cells to H₂O₂, the oxidative inactivation of mitochondrial ATP synthetase was examined (26). Lee et al. reported that epithelial cells of the inner ear coordinated their ion transport activity through the autocrine and paracrine signal pathway among neighboring cells in the ear via ATP (27). In addition, ATP is one of the commonly used medications for the treatment of MD in Japan (28). Our results demonstrated that the addition of ATP to H₂O₂-treated LCLs clearly recovered the viabilities in Me1, M3, M4, Me7, and Me10 cells, although the cells from Me2, Me5, Me6, Me8, and Me9 did not recover their viability after ATP treatment. Therefore, we thought that Me1, M3, M4, Me7, and Me10 cells were sensitive to ATP treatment, whereas Me2, Me5, Me6, Me8, and Me9 cells, by contrast, were not. Interestingly, as shown in Table 1, all ATP-sensitive cases were classified as AAO-HNS Stage 1 or 2 and all ATP-insensitive cases were classified as AAO-HNS Stage 3 or 4. After the ATP treatment, there was a significant difference (p < 0.01) of the viabilities of the H₂O₂-treated LCLs classified as Stage 1 or 2 and Stage 3 or 4 in MD (Figure 3). These results demonstrated that the sensitivity of H₂O₂-treated LCLs to ATP might represent a method for prognosis and treatment of MD. Clinically, some of patients staged 3 or 4 experiences poor control of vertigo, the progressive sensorineural hearing loss and the worsening of tinnitus even after several years treatment. The treatment of MD mainly aims to reduce these symptoms, because all three symptoms, either separately or in combination, cause great distress and have a considerable impact on the patients quality of life (29). Therefore, the prognostic expectation of MD is very profound for the quality of life of patients with MD. Additionally, these LCLs established from patients may be used for the drug susceptibility test in MD.

We also investigated morphological changes of the LCLs treated with H₂O₂. Figure 4 showed that after 5 h treatment, H₂O₂-treated LCLs showed either chromatin condensation (A) or swelling of the cell body (B).
In conclusion, LCLs established from MD patients could be used as a unique model to detect the susceptibility to oxidative stress and the effect of ATP treatment in MD patients. The difference of the sensitivity of H_2O_2-treated LCLs to ATP might relate to the prognosis of MD. This system may form the basis of tailor-made therapy for MD.

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References


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